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13. SUPPLEMENTARY NOTES

14. ABSTRACT The goals of the project were to assess the neuroprotective and antiepiletogenic properties of a mitochondrial-targeted antioxidant, SS-31 in the pilocarpine (PILO) model of status epilepticus (SE), the kindling seizure model and the tetanus toxin (Tx) model of epilepsy. Progress on the project has been limited throughout the grant period due to an inability to obtain a sufficient quantity of SS-31 to perform the proposed experiments. This resulted in a no cost extension granted in April 2014. Our last shipment of SS-31 was obtained on May 29, 2014. In this reporting period we completed the experiments proposed in Aim# 1, testing the efficacy of SS-31 as a neuroprotectant/antiepileptogenic agent in the PILO model of SE and as an antiepileptogenic agent in the kindling seizure model. The results from these experiments confirmed our preliminary results that SS-31 had no effect on the latency to SE and that there was no evidence of neuroprotection in hippocampal tissue stained for: Nissl, Fluoro-jade C, NeuN and heat shock protein. The insult generated by prolonged seizure activity appeared to be too severe for SS-31 to be effective. We observed these negative results despite testing SS-31 at dose of 10mg/kg. s.c., which is higher than what has been reported to be efficacious in other models. In Aim#2 we tested the efficacy of SS-31 in the kindling model. In our preliminary experiments a kindling stimulus was delivered to the hippocampus of each rat and the stimulus-induced afterdischarge (AD) threshold and duration were determined. A minimum of 24hr later SS-31 (10-20mg/kg, s.c.; n=9) was administered 30min before AD testing. SS-31 had no effect on AD threshold but there was preliminary evidence that SS-31 (20mg/kg) decreased AD duration in some animals. In this reporting period we repeated these experiments increasing the dose of SS-31 to 30mg/kg, s.c. (n = 5). At this dose SS-31 had no effect on AD threshold or duration. These experiments exhausted our supply of SS-31 such that we were unable to test the effect of SS-31 on kindling acquisition or complete the experiments in Aim#3. A second no cost extension was granted on May 14, 2015. Due to the failure of SS-31 to be effective in the PILO and kindling models and our inability to obtain more drug we will use the remaining funds to test the neuroprotective properties of carbonyl cyanide 4-trifluromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler in the PILO model of SE.

15. SUBJECT TERMS
Neuroprotection, antiepileptogenesis, antioxidant

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INTRODUCTION:
A number of studies have provided evidence that reactive oxygen species play a role in the induction of seizures and seizure-induced neuronal death. The goals of this project are to test the efficacy of a novel, mitochondrial-targeted antioxidant SS-31, as a neuroprotective and antiepileptogenic agent in three experimental models of epilepsy. The pilocarpine-induced model of status epilepticus (PILO) will be used to test SS-31 as a neuroprotectant, the kindling model will be used to test SS-31 as an antiepileptogenic and anticonvulsant agent, and the tetanus toxin model (TX) will be used to test SS-31 as an anticonvulsant. If SS-31 proves to be effective in these studies future experiments will test SS-31 in models of traumatic brain injury.

Body:
SS-31 was created by Dr. Szeto but the rights to the drug are controlled by Stealth Peptides, Inc. Progress on the project during the initial grant period was limited due to difficulty obtaining sufficient quantities of the test agent, SS-31 to perform the proposed experiments. This led to the granting of a no-cost extension in April of 2014. At the end of May 2014 the PI was able to acquire a new supply of SS-31. In the previous grant period we expanded our preliminary studies using the PILO model (Aim #1), and began testing SS-31 in the kindling model (Aim#2). In the current grant period we completed the experiments in Aim#1 and Aim #2. Unfortunately any hint of a positive effect that was observed in preliminary experiments was not confirmed when the experiments were repeated and the number of animals examined was increased. In the case of the experiments using the kindling model we increased the test dose of SS-31 to 30mg/kg, sc, a dose of SS-31 significantly higher than what has been reported to prevent cell death in models of cardiac ischemia (Cho et al., 2007), kidney ischemia (Szeto et al., 2011) and mechanical ventilation-induced diaphragm weakness (Powers et al., 2011). Testing the drug at this elevated dose still failed to reveal a positive effect but contributed to the exhaustion of our current supply of SS-31. Our continued difficulty in obtaining more SS-31 resulted in the granting of a second no-cost extension in May, 2015.As a result of the failure of SS-31 to be effective in the PILO and kindling models and our inability to obtain more drug we have begun testing the neuroprotective properties of carbonyl cyanide 4-trifluro-methoxy-phenylhydrazone (FCCP), a mitochondrial uncoupler in the PILO model of SE. The decision to test FCCP was agreed to by Captain Sawyer, DVM, the Scientific Officer for the project. We decided to test the neuroprotective properties of FCCP because it is commercially available and because it has been shown to be neuroprotective in an experimental model of traumatic brain injury (Pandya et al., 2009). The testing of a mitochondrial uncoupler is consistent with the theme of the proposal of developing a neuroprotective-antiepileptogenic therapy by preventing mitochondrial dysfunction.

Aim #1 – **Test the neuroprotective and anticonvulsant properties of SS-31 in the pilocarpine model of status epilepticus (SE) in the rat.**
In this model, prolonged seizure activity causes neuronal cell death in specific neuronal populations in the rodent hippocampus. Adult male Sprague-Dawley rats (260-405g) were used. In our initial experiments, experimental animals were pretreated with SS-31 (3 or 10mg/kg, sc) 45min before induction of SE with pilocarpine (365mg/kg, sc). One hour after the onset of SE each animal received an injection of diazepam (5mg/kg, ip) to attenuate SE and to improve survival. Control animals received an injection of saline instead of SS-31. To determine whether treatment with SS-31 affected the development of SE, the time to the onset of SE was measured for control and SS-31 treated animals. To examine neuroprotection the animals were perfusion-fixed with 4% paraformaldehyde (PAF) 1-3 days after SE. The brains were sectioned on a vibratome through the dorsal hippocampus. Sections were processed for the following histochemical and immunohistochemical stains: Nissl, Fluoro-jade C (FJ), NeuN and heat shock protein 70-72 (HSP). Nissl, FJ and NeuN stains were used to assess neuroprotection. HSP was used to detect neuronal stress but under some conditions has been shown to be neuroprotective. To pursue the possibility that PILO-induced SE was too severe an insult to observe a neuroprotective effect of SS-31 we also tested SS-31 in a modified version of the PILO model to limit the amount of PILO-induced seizure activity. In these animals we administered a higher dose of diazepam (10mg/kg, ip) 5min after the onset

of SE instead of the usual 60min. We also injected SS-31 into the experimental rats 30min before the injection of PILO instead of 45min.

Results from Current Grant Period - Aim #1
We confirmed our preliminary results that the administration of SS-31 (10mg//kg, sc) 30-45min before induction of SE with PILO (365mg/kg, sc) did not delay the onset of SE and that histochemical/immunohistochemical staining of hippocampal tissue 1-3 days after SE provided no evidence of neuroprotection in SS-31-treated rats. A total of 42 rats were evaluated in this Aim (17 controls and 25 experimental). Three rats from each group died during SE. Our conclusion from these results is that the insult induced by SE is too severe for SS-31 to be effective. An additional possibility is that pretreatment with SS-31 before the onset of SE is not the optimal window for drug delivery. It is possible that delivery of the antioxidant after the degenerative process has started would yield positive results. This approach will be investigated in future experiments using the mitochondrial uncoupled FCCP.

Aim #2 –Test the antiepileptogenic properties of SS-31 in the kindling seizure model. Kindling is a seizure model where repeated, spaced delivery of an initially subconvulsive stimulus to a limbic structure results in a permanent change in brain function such that eventually the kindling stimulus regularly elicits a limbic seizure. Progression through the kindling process can be assessed by measurement of the severity of the behavioral seizure and by measurement of the threshold and duration of the electrographic afterdischarge (AD). Behavioral seizures are scored on a 1-5 scale with stages 1-2 being equivalent to partial seizures and stages 3-5 equivalent to generalized convulsions. Once animals have exhibited 3 consecutive stage 5 seizures the kindling process is considered complete the animals are considered to be fully kindled. The number of stimulations required to reach a given stage, AD threshold and AD duration can be measured to assess the epileptogenic process. AD threshold is determined by delivering a kindling stimulus at a low current intensity. If no AD is detected the delivery of the kindling stimulus is repeated at a higher current intensity until an AD is elicited. AD testing is initiated at a current intensity of 5µA and increased in 5µA increments until an AD with a duration of at least 5sec is observed.

Effect of SS-31 on AD threshold and duration
Our first experiment was to determine if pretreatment with SS-31 altered AD threshold and AD duration. Each of the rats acted as their own control. Bipolar platinum depth electrodes were stereotaxically implanted bilaterally into the dorsal hippocampi of 13 anesthetized adult male Sprague-Dawley rats. Several screws electrodes were also implanted into the skull to allow for recording of surface EEG and to act as a ground. The electrodes were connected to a headstage which allows for connection to a stimulation/EEG recording system through a cable. Animals were allowed to recover from electrode implantation surgery a minimum of one week before entering into the study. The kindling stimulus is delivered through the same electrodes used to record EEG activity in an awake, freely-moving animal. Data were collected from 9/13 rats as 4 rats were removed from the study due to poor recording quality or loss of the electrode head stage. The experimental design consisted of initially determining baseline AD threshold and AD duration. A minimum of 24hr after the determination of baseline AD threshold and duration each animal was treated with SS-31, 30min before delivery of the kindling stimulus. This pattern was continued over a number of days. For this experiment the kindling stimulus had the following characteristics: 60Hz, 1msec biphasic pulse, delivered for 2sec.

Results from the current grant period – Aim #2
In the previous grant period we observed no effect of pretreatment with SS-31 (10mg/kg. sc) on AD threshold or AD duration (Figure 1). When we increased the dose of SS-31 to 20mg/kg, sc (n=9) there was still no effect on AD threshold (Figure 2A) but we did observe a decrease in AD duration in 6/9 rats (Figure 2B).

For the current grant period we repeated these experiments administering SS-31 at a dose 30mg/kg,sc (n=5). Unfortunately when SS-31 was tested at the higher dose we were unable to repeat the positive effect we observed on AD duration with a dose of 20mg/kg (Figure 3 A and B). Our inability to reproduce

the results we observed with 20mg/kg lessened our enthusiasm for the positive results we observed with 20mg/kg. Our difficulty in obtaining more SS-31 limits our ability to clarify these findings by repeating these experiments. It is important to recognize that the doses of SS-31 tested in the PILO and kindling models were significantly higher than what has been reported to be effective in other models of cell death. This raises the possibility that SS-31 does not readily crossing the blood-brain barrier.

Aim #3 – Test the anticonvulsant properties of SS-31 in the Tetanus Toxin (TX) model of mesial temporal lobe epilepsy. The combination of the negative results we obtained with SS-31 treatment in the PILO and kindling models and our continued difficulty in obtaining sufficient quantities of SS-31 led to the decision to replace the experiments proposed in Aim #3 testing SS-31 in the TX model with experiments designed to test the neuroprotective efficacy of carbonyl cyanide 4-trifluro-methoxy-phenylhydrazone (FCCP), a mitochondrial uncoupler in the PILO model of SE. We decided to test FCCP because it is commercially available and because it has been shown to be neuroprotective in an experimental model of traumatic brain injury (Pandya et al., 2009). The testing of a mitochondrial uncoupler is consistent with the theme of the proposal of developing a neuroprotective-antiepileptogenic therapy by preventing mitochondrial dysfunction. In order to perform these experiments a new ACURO Appendix had to be submitted and approved along with a modification adding the FCCP experiments. We have begun these experiments administering FCCP (2.5mg/kg, ip) 75min after the onset of SE. At this time we do not have any preliminary results to report.

KEY RESEARCH ACCOMPLISHMENTS:

- The experiments proposed in Aim #1 have been completed. Treatment with SS-31 (3-10mg/kg, sc) did not delay the onset of status epilepticus in the pilocarpine model..
- SS-31 was not neuroprotective in the pilocarpine model of status epilepticus
- For Aim #2 we previously reported SS-31 at a dose of 20mg/kg had no effect on AD threshold but shortened AD duration in 6/9 animals suggesting a positive effect on the epileptogenic process. During this reporting period we repeated the experiment increasing the dose of SS-31 to 30mg/kg. The higher dose of SS-31 did not replicate our earlier findings.
- We have replaced the experiments proposed in Aim #3 to test the efficacy of SS-31 in the tetanus toxin model with experiments testing the neuroprotective properties of FCCP, a mitochondrial uncoupler in the pilocarpine model. These experiments have been initiated and will be completed by the end of the project. FCCP is commercially available so access to the drug will interfere with the execution of these experiments.

REPORTABLE OUTCOMES:

None at this time.

CONCLUSIONS:

Progress throughout the project has been limited due to difficulty obtaining a sufficient quantity of SS-31 to effectively test its action. At the end of May, 2014 additional drug was received however in an attempt to obtain positive results, SS-31 was tested at significantly higher doses that what was originally proposed leading to a more rapid exhaustion of this most recent shipment. During this reporting period we completed the experiments proposed in Aims 1 and 2. Unfortunately we did not observe a positive effect of SS-31 on the latency to the onset of SE or anatomical evidence of neuroprotection in pilocarpine model of SE. Although the results from preliminary experiments indicated a positive effect of SS-31 (20mg/kg) on AD duration in kindling seizure model we were not able to reproduce these results when SS-31 was tested at 30mg/kg. The failure to obtain a significant positive effect in either seizure model suggests that the pilocarpine insult is too severe for this antioxidant to have a neuroprotective effect and raises the possibility that SS-31 does not cross the blood brain barrier as readily as originally anticipated. After discussions with the project's Scientific Officer it was decided that the direction of the project would be changed. The experiments originally proposed in Aim #3 would be replaced with experiments designed to

test the neuroprotective properties of FCCP, a mitochondrial uncoupler, in the pilocarpine model. FCCP has been reported to be neuroprotective in an experimental model of traumatic brain injury

REFERENCES: None

APPENDICES: None

SUPPORTING DATA:

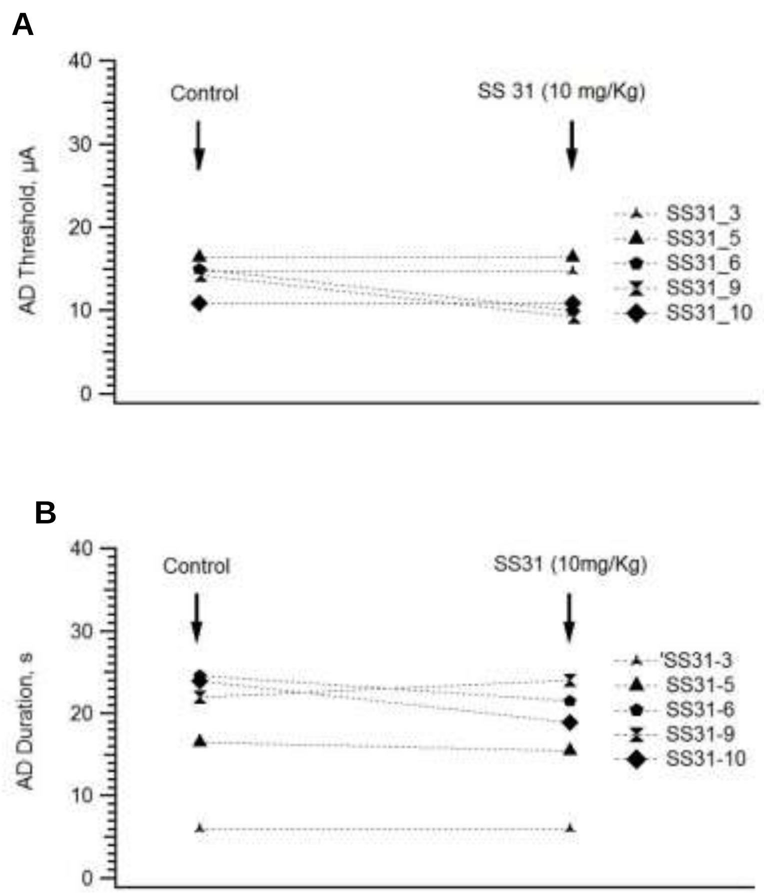

Figure 1 – Effect of SS-31 (10mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold or duration (n=5).

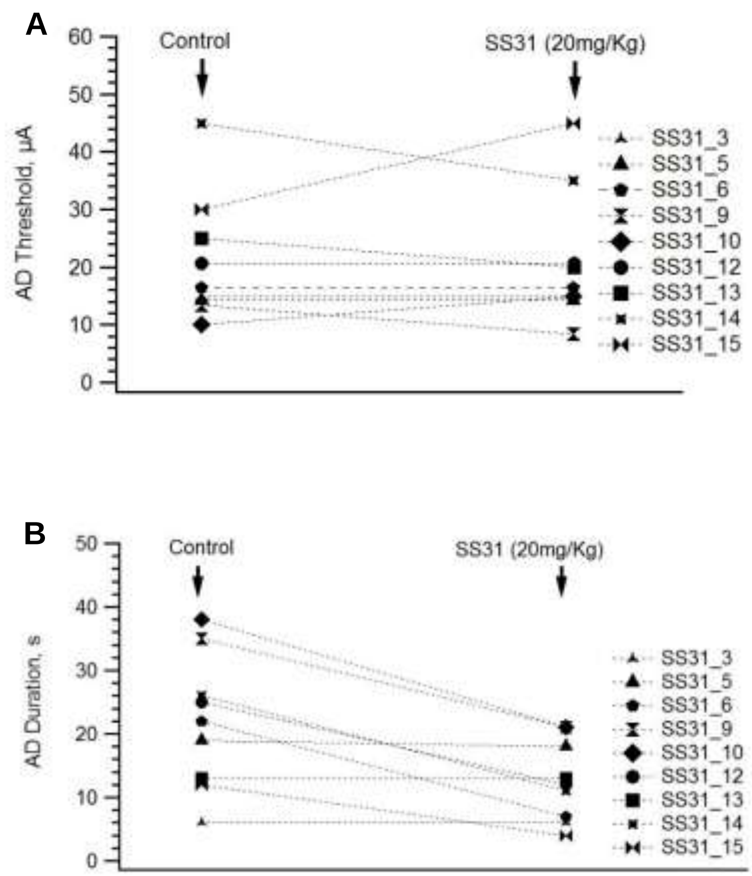

Figure 2 – Effect of SS-31 (20mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold but decreased AD duration in 6/9 animals (n=9).

A

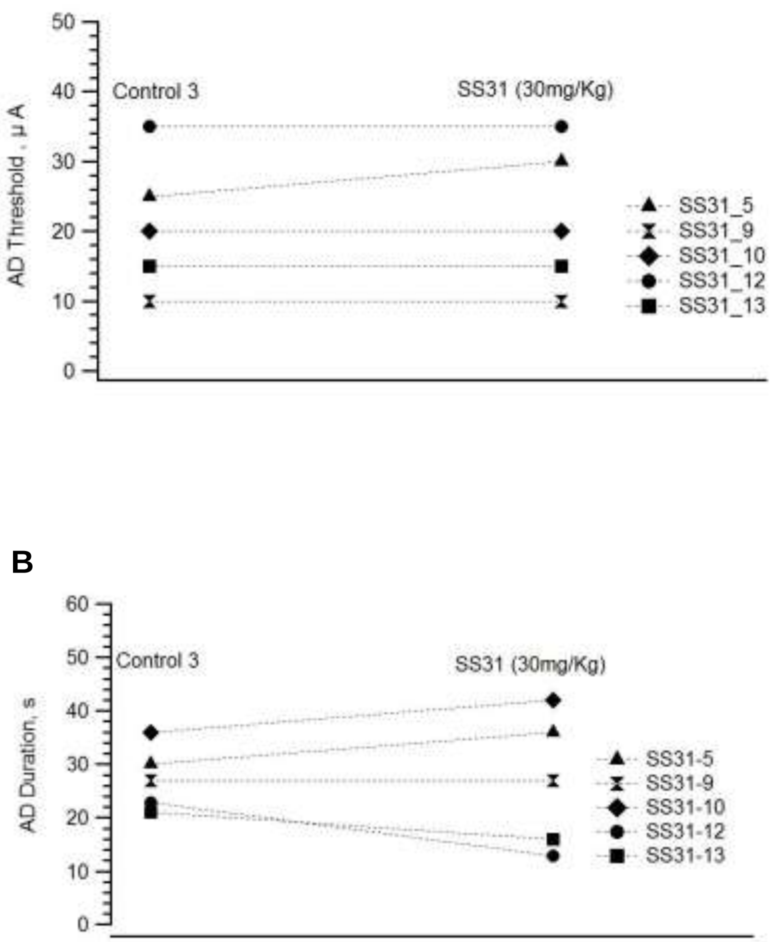

Figure 3 – Effect of SS-31 (30mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus did not have a significant effect on AD threshold or AD duration (n=5).